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Award Number: DAMD17-03-C-0111

TITLE: Analytical and Characterization Studies of Organic Chemicals, Drugs, and Drug Formulation

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REPORT DATE: March 2009

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
1 Mar 2009	Final	22 Sep 2003 - 21 Oct 2008

4. TITLE AND SUBTITLE

Analytical and Characterization Studies of Organic Chemicals, Drugs, and Drug Formulation

5a. CONTRACT NUMBER
DAMD17-03-C-0111

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

SRI International
Menlo Park, CA 94025-3493

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT Although the overall purpose of this contract was to perform chemical/physical analyses on bulk pharmaceutical substances and formulated drug products of interest to the USAMRMC Drug Development Program for parasitic and infectious diseases, chemical and biological defense, etc, by far the majority of time and effort expended during the current contract was devoted to design, development, and cGMP manufacture of an artesunic acid parenteral dosage form. Over 5,000 units of the two-component drug were released to the Army for clinical use. As a part of the cGMP manufacture, a program of stability studies was maintained over the entire contract period to ensure the continued integrity of the drug in its clinical use. To obtain genetox information on our drug, three major genetox assays were performed, and the reports were submitted to our COR. Attributes and faults of our product have been identified, and attempts have been made to correct these faults, in order to produce an improved drug. Associated with this effort were 108 reports, 7 certificates of analyses, one poster presentation, and one patent. Considerable time and effort were also spent on analysis and solutions stability studies on MMB-4, a promising nerve gas antidote of interest to the Army. Results from these studies merit the Army's continuing interest in developing MMB-4 into a drug product. Associated with the MMB-4 activities, four poster presentations and one paper were submitted for publication. In addition to the above activities, the core project team continued to serve all areas of the Army by performing chemical analyses when required, and reports on these analyses were submitted to the COR. Additionally, one paper based on work performed on the previous contract was published.

15. SUBJECT TERMS
Anti-Parasitic Drugs, Chemical Defense Agents, Chemical Analyses, Stability Studies, Formulation Development

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	13	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION

This final report for Contract DAMD17-03-C-0111 covers the period from September 22, 2003 to October 21, 2008. The report summarizes studies performed and lists the compounds/samples analyzed. The report also lists the personnel receiving pay from this effort and cites publications, meeting abstracts, and patents that resulted from this contract.

This contract was concerned with the analytical, characterization, and stability studies of chemicals, drugs and drug formulations. The work was monitored by Mr. William Y. Ellis, the Contracting Officer Representative (COR), Chief, Department of Chemical Information, Division of Experimental Therapeutics, Walter Reed Army Institute of Research (WRAIR).

The overall objective of this project is the operation of an analytical laboratory to determine the identity, purity, strength, quality, physical and chemical properties, and stability of bulk pharmaceutical substances and formulated drug products of interest to the USAMRMC Drug Development Program for parasitic and infectious diseases, chemical and biological defense studies, etc. Specific objectives are to design, develop, validate, and execute methods to determine the following characteristics of candidate bulk pharmaceutical substances and formulated drugs:

- Identity, purity, and strength
- Stability
- Other physical and chemical characteristics, including weight variation, content uniformity, and other such compendial requirements
- Qualitative and quantitative identity of impurities
- Special projects not covered by the above headings

FINAL REPORT

Activities Related to Artesunic Acid (Artesunate)

By far the largest amount of effort and time of the current contract was devoted to the development, production, and stability maintenance of the artesunate (AS) parenteral drug product, WR256283, its mannitol placebo, WR016506, and its phosphate dissolution medium, WR135946.

The two major obstacles encountered during the AS product development were sterilization of the active pharmaceutical ingredient (API) and the particulate counts in the constituted drug solution, USP <788>.

The generally recognized method of choice to produce a parenteral drug product is lyophilization (Lyo), which offers the advantages of facile sterilization and filling, gentle solvent removal, and rapid drug reconstitution. For all practical purposes, AS is water insoluble. Attempts to prepare a non-aqueous “lyo” product failed completely, owing to the difficulty in solvent removal and to the poor quality of the residual AS (lyophilizate). Because of its carboxylic acid function, AS dissolves ”readily” in aqueous bases; once in solution, the half-ester function in AS, formed by acylation of the hemiacetal in dihydroartemisinin (DHA), readily hydrolyzes, forming DHA, the pharmacologically active species that has limited solubility in aqueous media. Although artesunic acid dissolves readily in aqueous bases, the resulting solutions freeze quickly, and the lyophilization proceed smoothly, the residual “cakes” could not be obtained anhydrous, even under heroic conditions. The residual water in the lyophilizate continues to permit AS hydrolysis to produce DHA, which in turn results in failure of the USP <788> test; thereby ended our attempts to produce an acceptable AS lyo product.

Our next approach was to prepare a two-component drug product, a bulk API and a dissolution medium, which would be a pH 8 sodium phosphate solution. The first task was to devise a means to sterilize the API followed by unit filling under sterile conditions. The commonly available thermal and irradiation methods of sterilization failed to produce an acceptable material; all attempts ended in decomposition of the AS, albeit minor in most cases. Treatment with ethylene oxide (EtO) gave a sterile material that showed no detectable physical or chemical decomposition, was free of EtO and its hydrolysis products, and was acceptable for further development.

Using a modified, hand-operated commercial device that aspirates/discharges solid particles pneumatically, over 5,000 vials, each containing 110 mg of sterile AS, were filled. Although the filling process was manually laborious, all the fills were within the labeled specifications. Production of the phosphate dissolution medium was straightforward relative to the production of the API. Selection, sterilization, and filling of the drug placebo, mannitol, were also challenging and required considerable time and effort.

Among the numerous tests required for product release, the most critical was USP <788>, Particulate Matter in Injections. To pass this test, the reconstituted solution must contain less than 6,000 counts of ≥10µm particles per vial. Particles that are counted in our reconstituted solution include air bubbles, undissolved AS and DHA, lint, and other foreign matter. Foreign matter counts originating from the API itself and from the dissolution medium amounted to 100 counts or so; these low counts were essentially invariant and merited no further consideration. Counts from other sources, however, can easily cause test failure. After a great deal of development, including studies on rates of DHA formation and on apparent solubility of DHA in phosphate containing AS, along with rates of air bubble dissipation, we finally settled on a sample preparation protocol that enables the reconstituted drug product to pass USP <788> --- for the time being.

Dissolution of AS in an aqueous base requires wetting of the solid particles and agitation of the mixture. Once in solution, AS hydrolysis begins to form DHA. The optimum sample preparation procedure calls for sufficient agitation to quickly dissolve the API, while minimizing formations of DHA and air bubbles, as phosphate is an emulsifier. The air bubbles formed must dissipate before counting, and dissipation must occur while the solution is left undisturbed—all the while DHA continues to form. The developed sample prep allowed the drug product to pass USP <788>, and the product was released to the Army.

The development and production of the phosphate dissolution medium, second drug product component, was straightforward and required little or no extraordinary effort in the hands of experienced personnel.

The development and production of the drug placebo required several trials. The resulting placebo, mannitol, readily dissolves in phosphate, forms a stable solution, and offers no stability problem.

An integral part of the cGMP manufacture of the AS dosage form is the determination of stability under shelf-life and accelerated stress conditions. After storage of 6 months at 40°C/75% RH or 12 months at 25°C/60% RH, the product failed the USP <788>. The clinical supply of the drug, which had been stored at the Army repository at 18-21°C since receipt, was immediately transferred to -20°C storage and units pulled were analyzed for DHA content and particle counts. Although the unit DHA content has increased since product release, it was below the critical amount for precipitation. With modification made to the sample prep for the USP <788>, these units showed elevated counts over those found at the time of release; though the counts were near the failure limit, 6,000, nevertheless they passed.

A new shelf-life stability study with units stored at -20°C commenced, with units pulled every 3 months for stability determinations. The -20°C-stored clinical supply that is requested by clinician for use is sent to clinical pharmacies where the drug units are stored at +5°C until administered. For this reason, a second stability study with units stored at +5°C also started. Hence, essentially the entire remaining project effort was devoted to keeping the clinical supply stable and finding the cause of the product failure. The latter problem was particularly perplexing because bulk AS, whether or not treated with EtO, is known to be stable at room temperature for at least 4 years under shelf-life conditions, and the manufacturing of our product involved merely filling of the sterilized bulk drug. The manufacture filling process uses a device that aspirates and releases a metered mass of AS. The AS particles, while being aspirated and discharged, are in moving contact with a Teflon surface; this “rubbing” may have introduced a positive charge on these AS particles. A visual examination of the particles in the drug product showed suggestions of a static charge, although these suggestions were not striking. Such positive charges would attract the headspace water vapor to the charged particles and hasten AS hydrolysis. This speculation led to studies that include measurements of water vapor content in the vial headspace, while sealed units were kept in atmospheres of controlled humidity. A GC-based method capable of accurate measurement of relative humidity (RH) was developed, validated, and applied. Results from these studies showed sealed vials were not airtight and the RH in the vial headspace equilibrated with that of the controlled environment (chamber). Another meaningful study involved water vapor sorption and de-sorption on formulated and unformulated AS. Results from this study clearly showed that the formulated AS particles adsorbed water vapor faster and desorbed water vapor slower than the unformulated AS particles. Additional studies involved the use of non-glass containers with different closures were carried out, as were studies on the use of secondary containment. Results from the use of non-glass containers showed no significant stability improvement and the studies were discontinued. Studies that involved secondary containment proved the need to have cumbersome packaging; these studies also ended.

Because product failure due to high particulate counts in the re-constituted solutions requires ≤5% of the filled AS to be hydrolyzed, the amount of intact AS remaining, which would be in solution, would likely still satisfy the labeled requirement. If these particulates are removed by filtration, the filtrate could be use for administration. Moreover, if 0.2 µm filters were used, the filtrates could be assured of sterility. Anticipating such a situation, commercially available syringe filters that would remove the insoluble matters and produce sterile filtrates were tested. The resulting sterile filtrates easily passed the USP <788> test and showed quantitative AS recoveries.

Owing to possible physical/chemical changes to the AS particles during storage, and because of the subjective nature of agitating the mixtures during sample dissolution, the USP <788> sample preparation procedure required further modification to produce solutions that would pass the particulate counts test. Using these new modifications, units stored at least 24 months at -20°C and

units stored at least 28 months at +5°C have successfully passed the particulate test, thereby allowing the ongoing clinical trials to continue.

The above efforts on the AS dosage form and on its placebo produced 108 reports, seven (7) certificate of analysis, one poster, and one patent.

Another assignment related to AS was to consider/evaluate other methods producing a sterile bulk AS for future dosage form productions. The need for this study was necessitated by the following. Although EtO is commonly used to sterilize prosthetics and laboratory equipment, it is much less often used to sterilize bulk chemicals, for a number of valid reasons. Moreover, the institution that performed the last EtO treatment does not guarantee a sterile product after treatment. Should the next EtO treatment AS be non-sterile, production of the next batch of AS dosage form would be seriously hampered.

It is common knowledge that if an API is dissolved in an organic solvent and the solution is sterile filtered, the filtrate, including the solute, is sterile. If the sterile solute is recovered under sterile conditions, it would be sterile. Using this process, a sterile bulk AS can be obtained.

To gain an idea what solvents have been used to produce the Knoll and Guilin bulk samples of AS, we studied the residual solvents in each. Trace amounts of ethyl acetate, ethanol, methanol, heptane and pentane have been found. The two hydrocarbons are poor solvents for AS and are likely used to precipitate the AS from solutions in one of the other three. Methanol was not considered because of its higher toxicity than ethanol and ethyl acetate. Ethyl acetate was selected because of its lower possible reactivity with AS, although it can undergo trans-esterification with AS though less likely than ethanol.

Near-saturated solutions of AS in ethyl acetate were prepared by warming mixtures at 35°C to 40°C. These solutions were slowly added with stirring to 10 to 20 volumes of pentane or heptane chilled to 0°C. Immediate precipitation of AS occurred in all mixtures, and after continued stirring for about 20 min at 0°C, the precipitated AS was separated by centrifugation, followed by removal of the mother liquor by pipetting. The adsorbed solvents were removed by gentle blowing with nitrogen, followed by drying under high vacuum at room temperature. The AS obtained by this procedure persisted to contain a small amount (≤ 1%) of ethyl acetate, even upon reduction of particle size followed by further vacuum drying. Other than the residual ethyl acetate, the AS is essentially chromatographically identical to the starting material and the recovery percentage was at least 90. The AS that remained in the mother liquor, which also was essentially chromatographically identical to the starting material, can be recovered when the mother liquor is reused as a part of the solvent for another recrystallization. Evidence of trans-esterification would be manifested by DHA-acetate and/or ethyl succinate, both of which should be separable and detectable

by LC. Owing to the amount of AS found in the recrystallized material is essentially that of the starting material, the amount of AS that underwent trans-esterification would be very small, if any.

In theory, a recrystallization procedure, including filtration of the AS solution prior to recovery of the AS, can be done in a clean room, in which the dosage-unit-filling can also be performed. If sterility can be maintained throughout the operation, the final product should be sterile.

This series of experiments was halted temporarily until a suitable clean facility can be found to perform the entire operation, in order to show the end product is sterile.

Three genetox assays: “Evaluation of Artesunic Acid in the Mouse Bone Marrow Micronucleus Assay”, “Evaluation of Artesunic Acid in the Salmonella-Escherichia Coli/Microsome Plate Incorporation Assay”, and “Evaluation of Artesunic Acid in the Chinese Hamster Ovary (CHO) Chromosome Aberration Assay” have been carried out.

Activities Not Related to Artesunic Acid (Artesunate)

Though a much smaller task than the artesunate, analytical and stability studies on MMB-4, a bis(pyridinium aldoxime that the Army is evaluating to be a nerve gas antidote still consumed considerable time and effort. Extensively analyzed samples of MMB-4 dichloride and MMB-4-dimesylate by chromatography showed a common minor component among them. The minor and the major components have been isolated in pure state by chromatography, each characterized by NMR, and each equilibrated in solution to the same major/minor composition that was found in the starting material. The two component are geometric isomers; the major being more stable at room temperature. In neutral aqueous media, the major/minor ratio is approximately 98/2.

Efficacious treatment of nerve-gas poisoning is critically dependent on elapsed time between organophosphate exposure and treatment with an antidote; the shorter the time lapsed, the higher the probability of reactivating the phosphorylated enzymes. For this reason, self- or buddy-treatment is imperative. Automatic injectors that can administer the antidote solution intramuscularly are an effective means for providing rapid treatment. A critical requirement for antidotes administered by this route is solution stability. We have determined the pH at which MMB-4 is optimally stable and have conducted stability studies at this pH and at relevant temperatures over many months to obtain data that permit us to project times for 10% MMB-4 decomposition (t_{90}). The projected t_{90}

in years are ≥26 for storage at 25°C and ≥5 for storage at 35°C. These projected stabilities are good enough for self- or buddy-use of auto-injectors.

These activities on MMB-4 have resulted in three poster presentations and one paper submitted for publication.

Concurrent to all the activities already described, the contract core team continued to operate an analytical laboratory to serve all areas of the Army. Listed below are the samples analyzed, for which reports have been sent to the COR.

WR002976;BS04841, quinine sulfate

WR035928;BS9l1031, paromomycin sulfate

WR073633;BS88561, gentimicin sulfate

WR142490;BK11592, mefloquine

WR227825;BQ37522, a 2,4-diaminoquinazoline derivative

WR249655;B90463, HI-6 dimesylate

WR249943;BR52182 & BM04444, MMB-4 dichloride and dimesylate

WR253648;BQ36030, HS-6 dichloride

WR279393;BS88561 & BS88570 paromomycin/gentimicin cream

WR288901;BQ39531 a tetraacetyl derivative of WR227825

WR299958;BS81624 & BS88570, decoquinate

WR301797;BS81446, a N,N-bis(dichlorophenyl)diaminopyrimidinę

Presentations, Publications and Patents

A poster entitled “A cGMP Manufactured Artesunic Acid Dosage Formulation” was presented at the 2005 Asia Pacific Military Medicine Conference (APMMC).

A poster entitled “Characterization of MMB-4 Bulk Drug and Development of Its Dosage Formulation” was presented at the 2006 Bioscience Review.

A poster entitled “Impurity in the Nerve Gas Antidote MMB-4” was presented at the 2007 APMMC.

A poster entitled “Chromatographic Separation and NMR identification of the Minor Component in MMB-4” was presented at the 2008 APMMC.

A publication entitled “Enhancement of an Analytical Method for the Determination of Squalene in Anthrax Vaccine Adsorbed Formulations” has been published in Journal of Pharmaceutical and Biomedical Analysis 42 (2006) 494-499.

A paper entitled “Chromatographic Separation and NMR Characterization of Isomers in MMB-4, a bis(Pyridinium Aldoxime)” has been submitted to the Journal of Pharmaceutical and Biomedical Analysis for publication.

A US patent, “Methods for the Formulation and Manufacture of Artesunic Acid for Injection,” was granted in 2007; foreign patents have been applied.

Personnel Receiving Major Contract Support

A listing of personnel who received major contract support during the report period:

Peter Lim, P.I.
Ronald Spanggord, Assistant P.I.
Patrick Macauley, Chemist
Mindy Johnson, Chemist
Jennifer Wang, Chemist
Will Christman, Chemist II
Katherine Irwin, Chemist II
Manoj Maniar, Formulation Manufacturing Supervisor
Schridhar Hegde, Formulation Chemist II
Steve Kim, Formulation, Chemist I
Helen Parish, QC Supervisor
Esther Yau, QA Supervisor

CONCLUSION

The most significant accomplishment achieved during the current contract period was the design, development, cGMP manufacturing, and stability maintenance of an intravenous dosage formulation of artesunic acid. Over 5,000 units of the two-component dosage form was released and delivered to the Army; a fewer units of the artesunate placebo also was released and delivered to the Army.

As useful as the two-component drug may be, it has weaknesses in design and in shelf-life stability. Much time and effort were expended in defining these weaknesses and in developing means to overcome them. The identified weaknesses include the need to use ethylene oxide to sterilize the artesunate prior to filling. A more elegant and simpler method is to dissolve the artesunate in a sterile organic solvent, sterile filter the solution, and recover the sterile material via recrystallization. The approach is simple on paper but the availability of a sterile facility and sterile techniques are not. A second weakness is the unexpected short shelf-life of the artesunate, owing to its hydrolytic decomposition. Since hydrolysis requires water, then exclusion of water is the answer. Steps to achieve an anhydrous environment will be incorporated into the production of the next batch of artesunate.

The design and execution of the stability studies on the drug units provided evidence of artesunate integrity throughout its ongoing clinical trials.

Analytical and solution stability results on MMB-4 gave credence to its potential as a nerve gas antidote for self- or buddy-use.

Concurrent to conducting the above assignments, the overall project objective of the operation of an analytical laboratory to determine the identity, purity, strength, quality, physical and chemical properties and stability of bulk pharmaceutical substances and formulated drug products of interest to the USAMRMC drug development program for parasitic and infectious diseases, chemical and biological defense studies, etc was not neglected. Throughout the contract period, this laboratory continued to serve as a core resource to other areas of the Army when called upon.

Respectfully submitted,

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